Chondroitin sulfate is involved in lysosomal transport of lysozyme in U937 cells

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Accepted 27 October 2000
Journal of Cell Science 114, 345-352 © The Company of Biologists Ltd

SUMMARY

Human promonocytes U937 synthesize lysozyme and retain approximately one third of it within lysosomes. Lysozyme is not glycosylated; thus, it cannot be subject to mannose-6-phosphate-dependent targeting to lysosomes. It is a basic protein with a pI of 10.5 and is known to interact with negatively charged macromolecules like proteoglycans. Therefore, we examined whether the latter are involved in the lysosomal targeting of lysozyme in U937 cells. We partially diminished the electronegative charge of newly synthesized proteoglycans by inhibiting their sulfation with chlorate. This increased the rate of secretion of lysozyme. Upon treatment of U937 cells with phorbol esters, the rate of secretion of lysozyme was increased to more than 90%. This coincided with an almost complete redistribution of a [^{35}S]sulfate bearing proteoglycan to the secretory pathway. After a brief pulse with [^{35}S]sulfate in the control, 80% of the [^{35}S]sulfate-bearing proteoglycan was retained within the cells, whereas in the treated cells this proportion was decreased to 13%. The secreted proteoglycan was sensitive to chondroitinase ABC and bound to immobilized lysozyme. This interaction was disrupted by 50-300 mM NaCl. The intracellularly retained proteoglycan was degraded with a half-life of 50-60 minutes and seemed to be directed to lysosomes because in the presence of NH_4Cl the degradation was strongly inhibited. Our results suggest that the proteoglycan is involved in lysosomal targeting of lysozyme in U937 cells.

Key words: Lysozyme, Lysosomal targeting, Chondroitin sulfate, U937 cells, Sulfation, Chlorate

INTRODUCTION

The classical pathway of the transport of soluble lysosomal enzymes relies on the synthesis of mannose 6-phosphate residues, recognition of these by mannose 6-phosphate receptors and delivery of the receptor-bound phosphorylated glycoproteins to endosomal/lysosomal compartments (Dahms et al., 1989). It explains their segregation in mesenchymal and, partially, in other cell types. Since the delineation of this pathway, numerous lysosomal proteins have been reported to be transported to lysosomes independent of phosphorylation. Our knowledge on these alternative pathways, however, remains very limited. Glickman and Kornfeld (Glickman and Kornfeld, 1993) have reported on lysosomal transport of cathepsin D (CD) in lymphoblasts that depends on a protein motif. In the literature numerous examples of mannose 6-phosphate-independent targeting of lysosomal enzymes can be found and a few of these can be referred to here (Waheed et al., 1987; McIntyre and Erickson, 1993; Zhu and Conner, 1994; Nishimura and Himemo, 1995; Tikkanen et al., 1995; Nissler et al., 1998; Hiesberger et al., 1998). In a mouse model lacking both types of the mannose 6-phosphate receptors, cell type-dependent transport of various lysosomal enzymes has been observed (Dittmer et al., 1999). Lysosomal targeting that is clearly independent of any carbohydrate is exemplified by the non-glycosylated proteins such as cathepsin S (Nissler et al., 1998) and lysozyme (Radons et al., 1994). As a part of the nonspecific immune system, lysozyme (EC 3.2.1.17) degrades bacterial cell walls of gram-positive bacteria and the chitinous components of fungal cell walls. This enzyme is active over a broad pH range from 3 to 8 (Banerjee et al., 1973) and is suited to hydrolyze its substrates both within and outside cells. It is secreted by various epithelial cells and occurs in many body fluids, such as tears, saliva, milk and mucus, and helps to prevent colonization of epithelia and invasion of the system by bacteria. It is of interest that lysozyme is produced by chondrocytes, binds to glycosaminoglycans and appears to modulate the calcification of cartilage (Kuettner et al., 1975). Lysozyme is also produced by monocytes and macrophages that secrete a portion of the enzyme and retain the remainder within lysosomes (Ralph et al., 1976; Gupta et al., 1985). Apparently, these cells possess a transport system for the partial lysosomal delivery of lysozyme. This protein contains a surplus of basic amino acids and has a high isoelectric point of 10.5 (Fett et al., 1985). Its base character may explain its binding to negatively charged macromolecules such as DNA and proteoglycans, which has been reported by Steinrauf et al. (Steinrauf et al., 1999) and Kolset et al. (Kolset et al., 1996), respectively. Human promonocytes U937 synthesize a proteoglycan that is represented mainly by serglycin with chondroitin sulfate (CS) side chains (Uhlin-Hansen et al., 1993) and binds to lysozyme (Kolset et al., 1996). The interaction of lysozyme with serglycin is of interest because in several hematopoietic cell-types, proteoglycans are known to mediate delivery of positively charged proteinases, hormones and other molecules to secretory granules (Forsberg et al., 1999; Galvin et al., 1999).

In the present work we have investigated whether the proteoglycan of U937 cells is involved in the targeting of lysozyme to lysosomes. We treated U937 cells with $NaClO_3$, an inhibitor of sulfation (Baeuerle and Huttner, 1986) that is known to diminish the number of negatively charged groups in proteoglycans (Safaiyan et al., 1999) and examined its effect on the lysosomal targeting of cathepsin D and lysozyme. We used metabolic labelling of U937 cells with [^{35}S]sulfate to examine the turnover of sulfated proteoglycans. We also characterized the interaction of the labeled proteoglycan with lysozyme in vitro using human lysozyme that had been covalently attached to Sepharose Cl-4B.

MATERIALS AND METHODS

Materials and cells

Lysozyme was isolated from human milk according to Jollès and Jollès (Jollès and Jollès, 1967) and attached to CNBr-activated Sepharose Cl-4B according to the manufacturer´s instructions (Amersham Pharmacia, Freiburg, Germany). Chondroitinase ABC was obtained from Boehringer Mannheim (Germany). $NaClO_3$ was from Merck (Darmstadt, Germany). Pansorbin^{TM}, a cell wall preparation of *Staphylococcus aureus*, was from Calbiochem-Novabiochem (Schwalbach, Germany), 4β-phorbol 12-myristate 13-acetate (PMA) and all other reagents were from Sigma (Deisenhofen, Germany). Human promonocytic cells U937 (Sundström and Nilsson, 1976) were cultured in RPMI medium, containing 10% heat-inactivated fetal-calf serum (both from Gibco BRL, Eggenstein, Germany), 100 units/ml penicillin and 100 μg/ml streptomycin under air/CO_2 (19/1).

Metabolic labeling

Metabolic labeling was performed with Tran^{35}S-label (11-18 MBq/ml) or [^{35}S]sulfate (28.5-74 MBq/ml), purchased from ICN (Meckenheim, Germany) in methionine/cysteine – or sulfate – deficient RPMI medium, respectively. The labeling media contained 10% heat-inactivated fetal calf serum that was dialyzed against 0.9% NaCl. Sulfate-deficient RPMI medium contained 0.1 mg/ml ampicillin as an antibiotic. Before addition of the radioactive metabolite, 10^6 U937 cells were washed three times in the appropriate deficient RPMI medium and incubated in this medium for 0.5 hours and 2-3 hours prior to labeling with Tran^{35}S-label or [^{35}S]sulfate, respectively.

Immunoprecipitation

Rabbit antisera specific for human lysozyme and CD were raised in our laboratory. They were employed for immunoprecipitation as described earlier (Lorkowski et al., 1987) with the following modifications:

(1) Buffer A (lysis buffer) consisted of 50 mM Tris/Cl, pH 7.4, 0.9% NaCl, 0.5% Triton X-100, 1 mg/ml bovine serum albumin (BSA), 2 mM phenylmethanesulphonyl fluoride, 5 mM iodoacetamide, 1 mM $MgCl_2$, 20 μg/ml DNase. After the 15 minutes incubation at room temperature the lysate was cleared by centrifugation at 14,000 ***g*** for 2 minutes. Then, it was adjusted to 2 mM EDTA and 0.1% SDS.

(2) All washes of the immunoprecipitates were performed twice except the last wash, that was performed once with 50 mM Tris/Cl, pH 7.4.

Precipitation of macromolecules, affinity-chromatography with immobilized lysozyme and gel electrophoresis

Radioactively labeled secretions or cell lysates were adjusted to 10% (w/v) trichloroacetic acid (TCA) and kept on ice for at least 30 minutes. The precipitates were collected by 2 minutes centrifugation at 14,000 ***g***, washed three times with 0.5 ml ice-cold 5% (w/v) TCA and dissolved in either 90 μl reducing Laemmli sample buffer (followed by SDS-PAGE) or 0.45 ml 50 mM Tris/Cl pH 7.4 (followed by affinity-chromatography). In both cases the samples were neutralized with 1M Tris base. Lysozyme Sepharose Cl-4B columns were equilibrated with 50 mM Tris/Cl, pH 7.4, and allowed to bind secretory proteins from U937 cells for 15 minutes at room temperature. They were then washed extensively with the equilibration buffer until no radioactivity was detected in the eluate. The bound radioactivity was eluted with aliquots of the equilibration buffer that contained 0, 25, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450 and 500 mM NaCl. The contents of radioactively labeled polypeptides in the fractions were analyzed by liquid scintillation counting in a Tricarb liquid scintillation counter (Canberra-Packard, Dreieich, Germany) and by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). In the latter case, samples were mixed with fivefold concentrated reducing sample buffer and boiled for 5 minutes at 95°C prior to the separation. Separation of metabolically labeled and precipitated or immunoprecipitated polypeptides was performed by SDS-PAGE according to Laemmli (Laemmli, 1970). The separation was followed by a fluorographic detection (Laskey and Mills, 1975).

Degradation of proteoglycans by chondroitinase ABC

A digestion buffer (40 mM Tris/Cl, 40 mM Na acetate, 0.1 mg/ml BSA, 0.1% NaN_3, pH 8.0) was added to 7-18 μl of the secretory products from U937 cells to give a final volume of 50 μl. Chondroitinase ABC from *Proteus vulgaris*, 10 mU, was then added and the samples were incubated for 20 hours at 37°C. Controls were incubated in parallel in the absence of the enzyme. The incubations were terminated by adding TCA and the precipitable fractions were analyzed by SDS-PAGE and fluorography, or liquid scintillation counting as described above.

RESULTS

The lysosomal transport of lysozyme is selectively reduced by inhibiting biosynthetic sulfation

U937 cells were labeled with Tran^{35}S-label in the absence or presence of 50 mM $NaClO_3$, or 100 nM PMA for 2 hours and the label was chased for 5 hours in the continued absence or presence of the respective drugs. Immunoprecipitates of lysozyme from cell lysates and media revealed that approximately one third of the enzyme was kept within the cells (Fig. 1). Upon inhibiting biosynthetic sulfation with $NaClO_3$, the fraction of intracellular lysozyme was significantly reduced. A further reduction of the intracellular lysozyme contents was accomplished by treating cells with PMA. As reported earlier (Radons et al., 1994), PMA interferes with the sorting of newly synthesized lysozyme. Inhibition of sulfation had a similar, though less pronounced, effect on the transport of lysozyme.

To investigate whether transport of other soluble lysosomal enzymes can be altered by blocking biosynthetic sulfation in U937 cells, lysosomal targeting of CD was examined in the absence or presence of $NaClO_3$ and/or NH_4Cl. The latter salt is known to interfere with the M6P-dependent transport of lysosomal enzymes and to induce secretory release of newly synthesized lysosomal enzymes (Hasilik and Neufeld, 1980). The labeling of lysozyme and more pronounced that of cathepsin D with Tran^{35}S-label was diminished in the presence of 50 mM $NaClO_3$, therefore, in this experiment the concentration of $NaClO_3$ was reduced to 20 mM. Similar to the

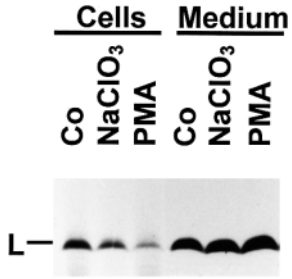

Fig. 1. Effects of $NaClO_3$ and PMA on the intracellular sorting of lysozyme in U937 cells. U937 cells were labeled with Tran^{35}S-label for 2 hours and the label was chased for 5 hours in the absence (Co) or continued presence of 50 mM $NaClO_3$ or 100 nM PMA. Lysozyme was immunoprecipitated from cell extracts and the medium, separated by SDS-PAGE with 5% and 15% acrylamide in the stacking and separating gels, respectively, and visualized by fluorography.

previous result, the secretion of lysozyme was increased. However, that of CD was not (Fig. 2). In contrast to the effect of $NaClO_3$, NH_4Cl increased the rate of secretion of newly synthesized CD, whereas it did not alter the secretion of lysozyme. These data imply that sulfation of macromolecules plays a role in the transport of lysozyme to lysosomes, but not of CD.

The major sulfated product in U937 cells is chondroitin sulfate

In order to characterize the products that are most affected by inhibition of biosynthetic sulfation U937 cells were labeled with [^{35}S]sulfate overnight in the absence or presence of 20 or 50 mM $NaClO_3$ or 100 nM PMA. Cells were then lysed and aliquots of cell lysates and the medium were subjected to precipitation with TCA, solubilized in reducing sample buffer, and analysis by SDS-PAGE and fluorography. Aliquots of control and PMA-induced secretions were digested with chondroitinase ABC and analyzed as well.

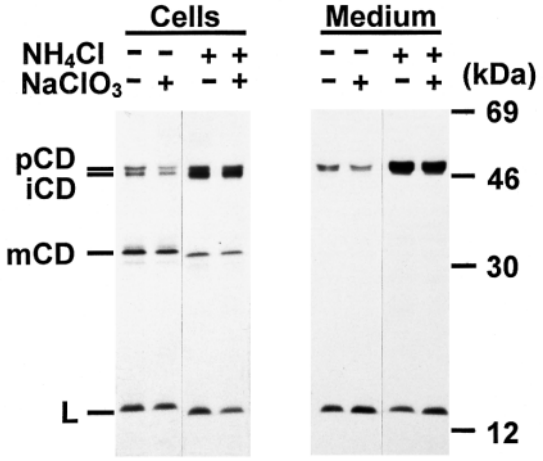

Fig. 2. Influence of NH_4Cl and $NaClO_3$ on lysosomal transport of lysozyme and CD. U937 cells were subjected to a pulse/chase labeling (as described in Fig. 1) in the absence or continued presence of 20 mM $NaClO_3$ or 10 mM NH_4Cl, and lysozyme and CD were simultaneously immunoprecipitated from extracts of cells and the medium. The immunoprecipitated material was analyzed as described in the legend of Fig.1. The positions of lysozyme (L), procathepsin D (pCD), intermediate CD (iCD) and mature CD (mCD) are indicated on the left. The positions of molecular mass markers are indicated on the right.

Most of the radioactive sulfate was incorporated into a high molecular mass species (M_r≥330,000), a large portion of which remained within the stacking gel (S in Fig. 3). Treatment of cells with $NaClO_3$ almost completely blocked incorporation of radioactive sulfate into these macromolecules (Fig. 3).

A large portion of the high molecular sulfated material was found in the medium, whereas a smaller portion was associated with the cells. In the presence of PMA, secretion of these molecules was significantly enhanced at the expense of the intracellular fraction. Chondroitinase ABC digestion of the secreted material released 95% of the radioactive sulfate from the precipitable fraction, indicating that it consists mainly of CS-bearing proteoglycans. The residual degradation products had molecular masses slightly higher than 30 kDa (labelled D in Fig. 3). The nature of these products was not examined. U937 cells that were labeled with Tran^{35}S-label produced similar macromolecular species that were sensitive to chondroitinase ABC. This treatment yielded heterogeneous degradation products in the range of 17-34 kDa (not shown). We have not attempted to identify the sulfated macromolecules, since it is known that the major proteoglycan of U937 cells is serglycin (Uhlin-Hansen et al., 1993) with a molecular mass of the polypeptide backbone including the signal sequence of 17.6 kDa (Stevens et al., 1988). We assume that in our experiments the treatment with chondroitinase ABC did not remove all carbohydrate from its protein backbone.

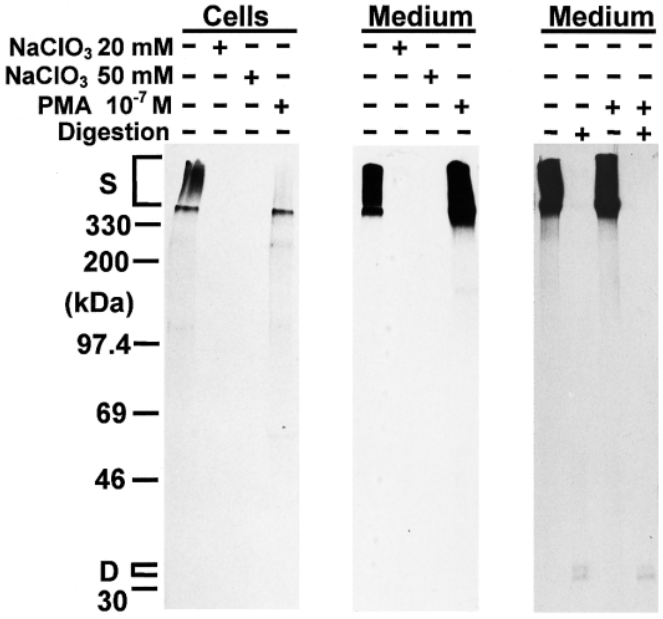

Fig. 3. Metabolic labeling of U937 cells with [^{35}S]sulfate in the absence or presence of $NaClO_3$ and PMA. Sensitivity of [^{35}S]sulfate -labeled secretory proteoglycans to chondroitinase ABC. Cells were labeled with [^{35}S]sulfate overnight in the absence and presence of 20 or 50 mM $NaClO_3$ or 100 nM PMA. Aliquots of extracts of cells and the media (1/2 volume) were precipitated with TCA and analyzed either directly (left and middle) or after an incubation with or without chondroitinase ABC (right). The radioactivity was analyzed by SDS-PAGE with 5% and 10% polyacrylamide in the stacking and separating gels, respectively, and fluorography. The position of the stacking gel (S), molecular mass markers and degradation products of the chondroitinase ABC-digests (D) are indicated on the left.

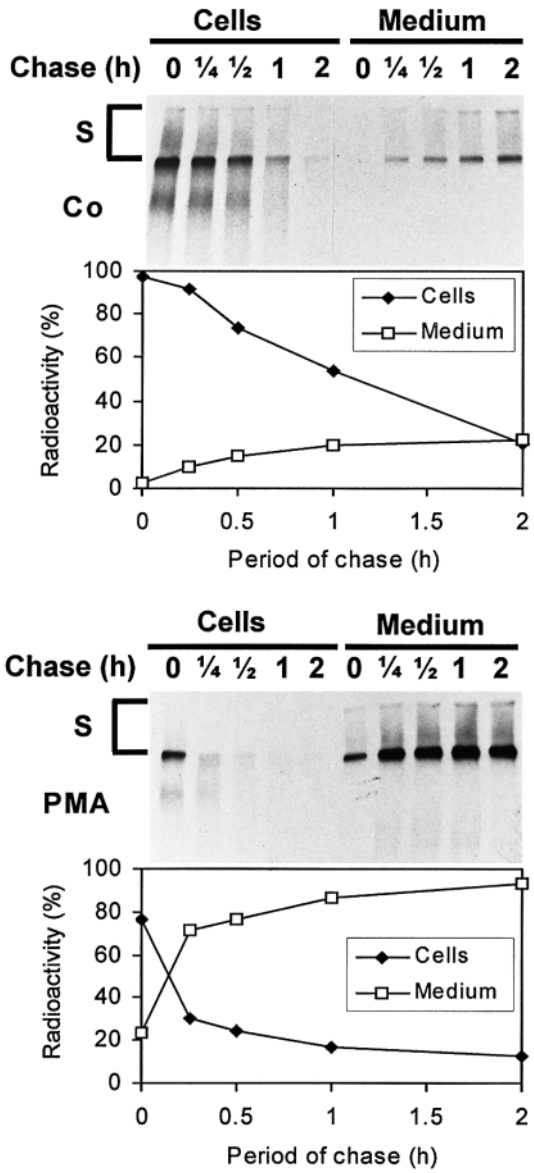

Fig. 4. Secretion of CS is greatly stimulated by PMA. U937 cells were labeled with [^{35}S]sulfate for 10 minutes and chased for up to 2 hours in the absence (top) or presence of 100 nM PMA (bottom). TCA-precipitable polypeptides from cell lysates and media were then analyzed by SDS-PAGE as described in the legend of Fig. 3 and liquid scintillation counting. Values are given as percent of the initial TCA-precipitable radioactivity and represent the mean of two determinations.

The apparent secretion of chondroitin sulfate is greatly stimulated by PMA

In order to examine the secretory release of CS-containing proteoglycans more closely, U937 cells were labeled for 10 minutes with [^{35}S]sulfate and the label was chased for up to 2 hours. Polypeptides from corresponding aliquots of cell-lysate and the medium were precipitated with TCA and analyzed by liquid scintillation counting and SDS-PAGE. Like in the preceding experiment, a large majority of [^{35}S]sulfate was incorporated into high molecular mass products that were found in the stacking gel and at the top of the separation gel.

In untreated cells, approximately 20% of CS was secreted within a period of two hours (Fig. 4). The maximum secretion was reached within approximately one hour of chase. During the 2 hours chase period the contents of intracellular CS dropped to approximately 20% of the initial radioactivity indicating a rapid degradation. After treatment of cells with PMA, secretion of CS was strikingly increased as compared with the control. This increase coincided with a rapid depletion of intracellular CS. Similar to the control, within 1 hour of chase the secretion of CS was close to its maximum (approximately 90%). Apparently, the treatment enhanced the rate of secretion of CS. As a result, its degradation was prevented. In conclusion treatment of U937 cells with PMA caused an almost complete secretion of both CS and lysozyme.

Intracellular chondroitin sulfate is subject to a rapid degradation

Next, we examined the possibility that the intracellular retained CS is targeted to lysosomes and degraded. It was assumed that in this case the degradation would be inhibited in the presence of NH_4Cl, which is known to raise the pH in the lysosomal compartment. U937 cells were labeled with [^{35}S]sulfate for 70 minutes and the label was chased for 0, 0.5, 1 and 2 hours in the absence or presence of 10 mM NH_4Cl. The incorporation of [^{35}S]sulfate into TCA-insoluble macromolecules was then

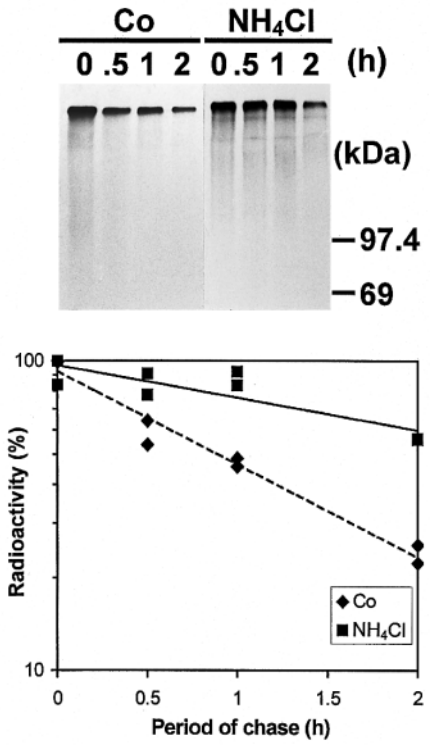

Fig. 5. Pulse/chase labeling of U937 cells with [^{35}S]sulfate in the absence and presence of NH_4Cl. After a pulse labeling with [^{35}S]sulfate for 70 minutes U937 cells were chased up to 2 hours. The pulse and chase media contained 10 mM NH_4Cl in one set of cells. Macromolecules were precipitated with TCA from corresponding aliquots of cell-extracts and media, and analyzed by SDS-PAGE and fluorography (top) with 3% and 10% polyacrylamide in the stacking and separating gels, respectively, or by liquid scintillation counting (bottom). Values are given as percent of the initial intracellular TCA-precipitable radioactivity.

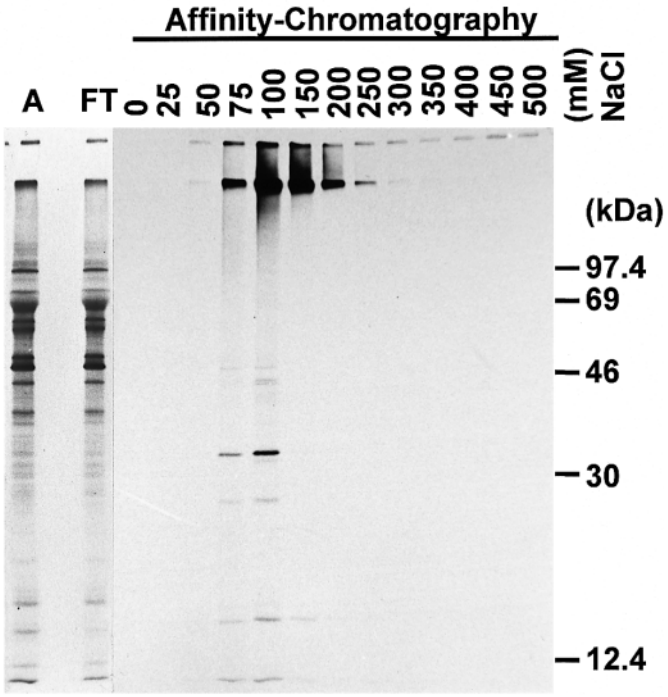

Fig. 6. Affinity chromatography of Tran^{35}S-labeled secretory products from U937 cells with immobilized human lysozyme. Conditioned medium of U937 cells that were labeled with Tran^{35}S-label overnight was subjected to precipitation with TCA. The sediment was dissolved and applied to a Sepharose Cl-4B column, containing covalently linked human lysozyme. The bound material was eluted by a discontinuous NaCl gradient and analyzed as described in the legend of Fig. 1. Corresponding aliquots of the starting material (A) and of the flow-through fraction (FT) were also examined.

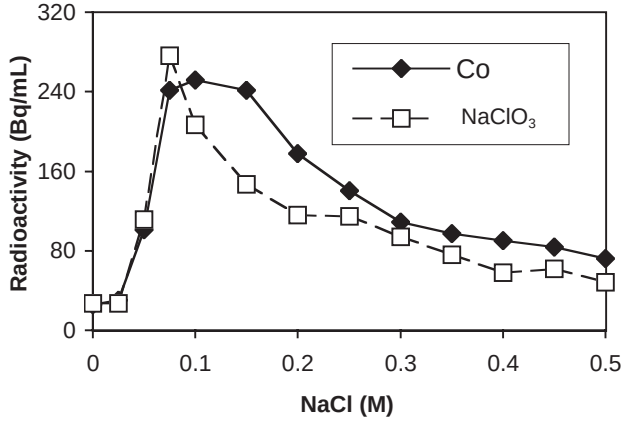

Fig. 7. Affinity chromatography of secretory products from U937 cells after a pulse/chase labeling with Tran^{35}S-label in the absence and presence of $NaClO_3$. U937 cells were labeled for 2 hours with Tran^{35}S-label in the absence or presence of 20 mM $NaClO_3$ and the radioactive label was chased for 5 hours in the continued absence or presence of $NaClO_3$. Aliquots of the secretory products were precipitated by TCA and applied to an affinity chromatography column with human lysozyme attached to Sepharose Cl-4B. The radioactive material was eluted using a discontinuous NaCl gradient and the fractions were analyzed by liquid scintillation counting. The apparent recovery of the radioactive products from $NaClO_3$-treated cells was 82% as compared with products of control cells.

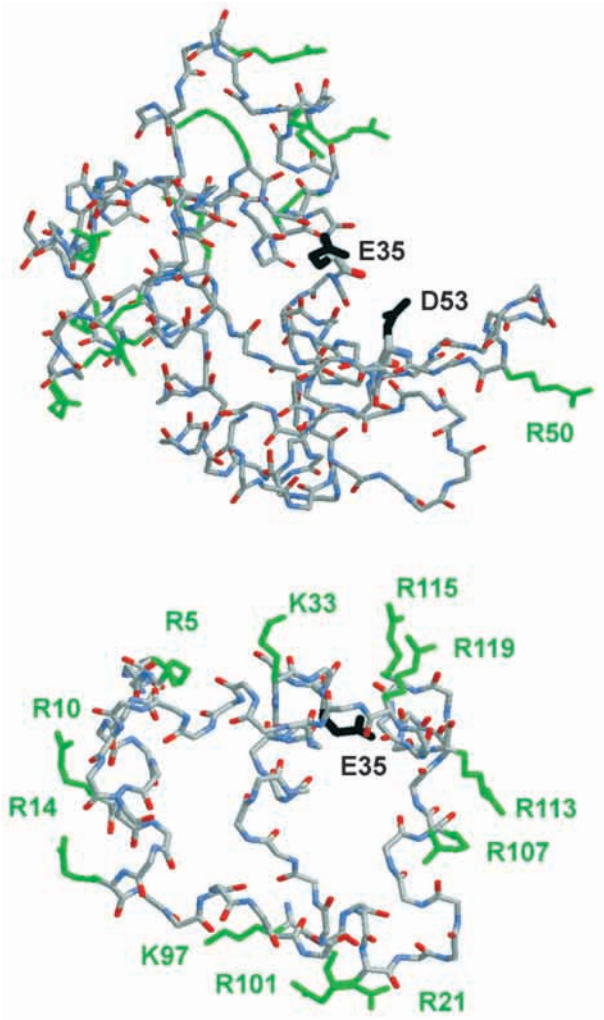

Fig. 8. Position of basic residues in the molecular model of human lysozyme. The upper part shows that all positively charged amino acids, except Arg50 are localized in one lobe of the molecule. In the lower part, the amino acid residues 1-36 and 97-130 with the ring-like arrangement of basic residues are shown. The side chains containing an uncompensated positive charge are shown in green and for comparison the side chains of Glu35 and Asp53, which comprise the catalytic center are in black. For the sake of clarity, the side chains of all other amino acids are not shown. The data were taken from the Brookhaven data bank and viewed by the Ras-Mol programme. The identity of amino acids is given in the one letter code.

determined by liquid scintillation counting, and the labeled material was analyzed by SDS-PAGE and fluorography (Fig. 5). Using a stacking gel with lower polyacrylamide-content (3% instead of 5% w/v), the labeled proteoglycans migrated and appeared as a relatively sharp band at the top of the separating gel. A characteristic downward 'smear' indicated the presence of proteoglycans with shorter CS chains.

The half-life of the labeled CS was 50-60 minutes (lower panel of Fig. 5). Treatment of U937 cells with NH_4Cl stabilized the intracellular CS. Thus, it is likely that this portion of CS is delivered to lysosomes shortly after its synthesis. During the 2 hours chase period 13 and 18% of the TCA-precipitable [^{35}S]sulfate-labeled material was secreted by NH_4Cl-treated

and control cells, respectively, indicating that loss of TCA-precipitable [^{35}S]sulfate labeled CS was a consequence of degradation rather than secretion (for comparison, see Fig. 4). In the presence of NH_4Cl the incorporation of [^{35}S]sulfate into CS was decreased by approximately 50%. Therefore, exposure times of X-ray films were adjusted to give comparable signals. Subcellular fractionation experiments with sucrose density gradients confirmed that the degradation of the [^{35}S]sulfate labeled proteoglycan was associated with lysosomes (not shown).

Interaction of lysozyme with CS

To directly probe binding of CS to lysozyme we concentrated CS from secretions of U937 cells that were labeled for 16 hours with Tran^{35}S-label by precipitation with TCA. The sample was dissolved, neutralized and applied to a lysozyme Sepharose Cl-4B column. After an incubation for 15 minutes at room temperature and extensive washing, the column was eluted with a NaCl-gradient and the fractions were analyzed by SDS-PAGE and fluorography (Fig. 6).

One major, high molecular weight polypeptide and several minor polypeptides were eluted from the lysozyme column. The major bound material resembled the [^{35}S]sulfate-labeled CS that was described above (Fig. 3). It had a similar high molecular mass and was sensitive to chondroitinase ABC-digestion (not shown). Elution was accomplished with a salt-gradient between 50 and 300 mM NaCl. Minor components co-eluting with CS consisted of several bands in the range of 14-50 kDa and at the migration front. We did not identify these polypeptides nor did we examine whether they were bound to lysozyme directly or via CS.

Binding of CS from control and $NaClO_3$-treated U937 cells to lysozyme

U937 cells were labeled with Tran^{35}S-label for 2 hours in the absence or presence of 20 mM $NaClO_3$ and the radioactivity was chased for 5 hours after adding an excess of non-radioactive cysteine and methionine (final concentration 0.15 mM, each). Aliquots of medium samples were subjected to precipitation with TCA and affinity chromatography as described in the preceding experiment. The results are shown in Fig. 7. Most of the radioactively labeled polypeptides from control secretions were eluted between 50 and 300 mM NaCl (diamonds in Fig. 7). In contrast, CS from $NaClO_3$-treated U937 cells was eluted at a lower ionic strength (squares in Fig. 7). In addition, the total amount of ^{35}S-labeled polypeptide binding to the lysozyme column was 82% (mean of two analyses), of control, indicating that sulfation of CS supports but is not indispensable for its binding to lysozyme. The other part of the binding was probably contributed by glucuronic acid which was not affected by the $NaClO_3$ treatment. This result confirms that CS-mediated lysosomal transport of lysozyme can be only partially inhibited when $NaClO_3$ is used to inhibit sulfation.

DISCUSSION

In this study we provide evidence that in human promonocytes, CS is involved in the lysosomal transport of lysozyme. In these cells, a small portion of CS proteoglycan is secreted, whereas the bulk is directed to lysosomes. The lysosomally targeted CS proteoglycan is degraded with a half-life of 50-60 minutes. In U937 cells, the major CS proteoglycan has been shown to consist of serglycin (Uhlin-Hansen et al., 1993). Therefore, the present results suggest that in U937 cells, the lysosomal targeting of lysozyme is mediated by CS of serglycin. This hypothesis is supported by the following observations:

(1) Lysozyme binds CS that is released at 50-300 mM NaCl, indicating that at physiological ionic conditions the binding is reversible and a significant fraction of lysozyme may be associated with CS.

(2) A major portion of CS (approx. 80%) is directed to the lysosomal compartment, where it is rapidly degraded.

(3) Inhibition of the sulfation of CS weakens the ionic interactions between CS and lysozyme and impedes the lysosomal transport of lysozyme.

(4) PMA induces a rather complete secretion of both CS and lysozyme.

A similar targeting mechanism of lysozyme may operate in human macrophages, which represent the fully differentiated state of monocytes. In human macrophages the major sulfated proteoglycan is a CS-bearing proteoglycan, most likely serglycin, 78% of which is targeted to lysosomes where it is rapidly degraded. These cells, when treated with the bacterial lipopolysaccharide, strongly increase the secretion of newly synthesized CS-bearing proteoglycans (Uhlin-Hansen et al., 1993). This effect is similar to the stimulation of the secretion of ^{35}S-labeled proteoglycans with PMA in U937 cells described here.

In rat basophilic leukemia cells, proteoglycans are directed mainly to intracellular storage granules and their secretion is strongly enhanced by PMA (Baldassarre et al., 2000). The effect of PMA indicates that a PKC signalling system may be involved in defense and inflammatory responses that mount in a localized release of lysozyme and other antimicrobial agents or signalling molecules. In fact, it has been shown recently that synthesis and secretion of macrophage inflammatory peptide-1α (MIP-1α) is severely increased after stimulation of U937 cells with PMA (Kolset et al., 1996). Interestingly, MIP-1α is one of the CS-binding proteins in U937 cells, indicating that CS is a multivalent carrier that may modulate several inflammatory and defense reactions. This notion is supported by our affinity-chromatography data (see Fig.6) showing either a direct or a CS-mediated binding of several polypeptides to the lysozyme-affinity column.

The binding of human lysozyme to CS from U937 cells resembles the binding of human and hen egg lysozyme to components of bovine cartilage, including CS and hyaluronic acid, that has been described by others (Moss et al., 1997). In cartilage, the contents of lysozyme are maximal in the hypertrophic zone adjacent to the site with the highest rate of synthesis of sulfated proteoglycans (Schmidt et al., 1978). Unlike monocytes, in chondrocytes the proteoglycans are predominantly secreted and it can be speculated that chondrocytes secrete both proteoglycans and lysozyme. In cartilage lysozyme is likely to modulate the calcification (Kuettner et al., 1975) and through its basic residues it may compete with growth factors for the acidic components of the matrix. We have examined the position of uncompensated positively charged amino acids (absence of a negative charge within a radius of 5 Å) in the known crystal structure of human

lysozyme (Artymiuk and Blake, 1981). We find that, except for Arg50 these residues are forming a ring on the surface of one of the two lobes of the molecule (Fig. 8). The ring involves arginine residues 5, 10, 14, 21, 101, 107, 113, 115 and 119, and lysine residues 33 and 97. This alignment of positive charges may favor a multivalent attachment of CS chains. Lysozyme is likely to interact with matrix components, also through its substrate binding site, since its effects on proliferation can be abolished by chitotriose, a specific inhibitor of the enzyme (Norton, 1982). Thus, lysozyme may affect the architecture and the function of the matrix by simultaneously binding several of its components.

As compared with the transport of other soluble lysosomal enzymes such as CD that of lysozyme is unique. It is inhibited when sulfation of CS is impeded by $NaClO_3$, whereas it is insensitive to NH_4Cl, an inhibitor of the M6P-dependent targeting. Thus, it seems to represent a transport pathway that is specialized for molecules bearing multiple positively charged residues. A similar pathway was shown to be used in mast cells, in which synthesis of sulfated heparin is indispensable for targeting of the mast cell proteinases into the granules (Forsberg et al., 1999).

In spite of the distinction mentioned above, there is some resemblance between the classical mannose 6-phosphate recognition system (Dahms et al., 1989) and the CS-mediated lysosomal transport of lysozyme. Both rely on Golgi-associated post-translational modifications that involve sugar moieties. In the case of CS-mediated lysosomal transport, it is the addition of glucuronic acid and the sulfation of N-acetylgalactosamine that occur in the medial- and trans-Golgi compartment (Sugumaran et al., 1998; Bäuerle and Huttner, 1987). This suggests that diversion of lysozyme from the secretory route takes place at or distal to this site. In order to understand this phenomenon more deeply, future efforts should concentrate on the mechanism of the lysosomal targeting of CS or other proteoglycans.

We greatly acknowledge the technical assistance of Silke Werth and Eva Becker, and the photographical work of Gertraud Jarosch. We are greatful to Evelin Heinemann for conducting the computer assisted artwork, Irena Müller for typing the manuscript and Dr Hans-Gerd Löffler for help in examining the arrangement of basic groups in lysozyme. This work was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 286) and the Fonds der Chemischen Industrie.

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